

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In Re Application of: David MUNN et al. Confirmation No.: 1273  
Serial No.: 10/780,150 Group Art Unit: 1614  
Filed: February 17, 2004 Examiner: Timothy P. Thomas

FOR: **REGULATION OF T CELL-MEDIATED IMMUNITY BY D ISOMERS OF INHIBITORS OF  
INDOLEAMINE-2,3-DIOXYGENASE**

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**Mail Stop Amendment**  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**DECLARATION OF DR. MARIO MAUTINO**

I, Mario Mautino, hereby declare that:

1. I am the Vice-President of Drug Discovery at NewLink Genetics, located in Ames, Iowa. NewLink is the exclusive licensee of the subject supplication. I hold a Ph.D. in Biological Chemistry and Molecular Genetics, received from the National University of Cordoba, Argentina in 1992. I have worked as a laboratory scientist in the fields of Molecular Genetics, Gene Therapy, Genomics, Immunology and Pharmacology, for a combined period of 17 years. I have published 22 peer reviewed journal articles and am a co-inventor in 6 patent applications, 3 of which are directed to novel inhibitors of indoleamine-2,3-dioxygenase (IDO). I have been involved in the discovery and testing of IDO inhibitors for the past 3.5 years leading all aspects of drug discovery and development in this area. I am familiar with scientific progress and various developments in the field of IDO-mediated immunoregulation. Therefore, I can be considered an expert in the field inhibitors of IDO.

2. I have read and am familiar with the contents of the Office Action mailed on June 12, 2009, for the application 10/780,150. I understand that the Examiner has rejected claims 2, 98-101, 103, 105-106, 108, 124-127, 129, and 131-132 under 35 U.S.C. § 112, first paragraph, as being not enabled by the specification. The purpose of this Declaration is to

provide factual evidence that administering compositions consisting essentially of 1-D-methyl-tryptophan (D-1MT) is effective in delaying the relapse or progression of a tumor in a subject when administered alone, without additional chemotherapeutic agents.

3. Attached as Exhibits A are graphs presenting data, which demonstrate the anti-cancer effects of the administration of D-1MT in a treatment regimen against lung cancer. Attached as Exhibit B is a paper by Hou, *et al.*, (2007) "Inhibition of Indoleamine-2,3-Dioxygenase in Dendritic Cells by Stereoisomers of 1-Methyl-Tryptophan Correlates with Anti-tumor Response," *Cancer Res.* 67(2):792-801. ("Hou")

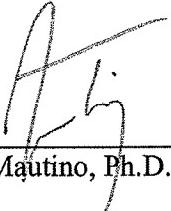
4. The data presented in Exhibit A was obtained as follows. B16Bl/16 mice were injected with  $3 \times 10^5$  LLC lung tumor cells subcutaneously (SC) on day 1. On day 5, 400 mg/kg of D-1MT was administered perorally (PO) twice daily (BID) Monday through Friday for 3 weekly cycles. Survival of the mice was monitored throughout the experiment and plotted in the survival curve of Exhibit A. Additionally, tumor volume was measured every other day for the duration of the experiment. As shown by the data in Exhibit A, the administration of D-1MT effectively prolonged survival in mice as compared with control treated mice (page 1). Furthermore, treatment with compositions consisting essentially of D-1MT also significantly reduced tumor volume compared to control treated mice (pages 2 and 3). From these data, I conclude that administration of D-1MT, by itself, is effective in prolonging survival and decreasing tumor volume in a lung cancer model.

5. I have read and understand the article by Hou, *et al.*, attached hereto as Exhibit B. The inventors of the present application are co-authors of the Hou article. The data presented in Hou, specifically that of Figure 5C, clearly shows that D-1MT is effective, without the addition of a chemotherapeutic agent, to decrease the tumor size of a melanoma tumor. In the experiment depicted in this figure, mice were injected with  $1 \times 10^6$  B78H10-GM-CSF tumor cells. It is my understanding that this cell line was derived from the B16F10 melanoma tumor cell line and has been altered to transgenically express GM-CSF, thereby rendering the resulting tumors more immunogenic. On the other hand, this cell line induces ~ 10-fold

higher number of immunosuppressive IDO+ dendritic cells in the tumor draining lymph nodes. Mice injected with these tumor cells were subsequently treated beginning on day 7, with timed release subcutaneous pellets of D-1MT at a dose of 5 mg/day. It is my understanding that this dose is 4 times less than that of the racemic DL-1MT given in previous experiments, for example depicted in Figure 1 of Hou. Figure 5C clearly shows that mice treated with D-1MT alone, had significantly smaller tumors compared with vehicle treated controls. I understand this data to indicate that D-1MT alone, without an additional chemotherapeutic is effective in reducing tumor volume in this melanoma tumor model.

6. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made based on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the above-referenced application or any patent issued therefrom.

9/10/09  
Date

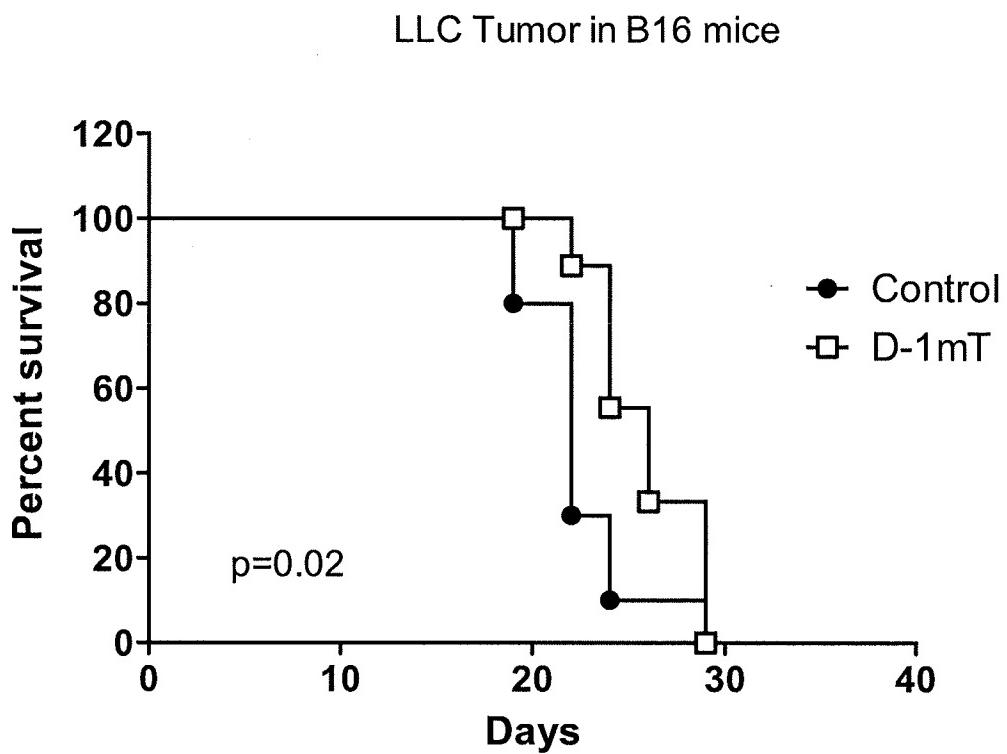
  
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Mario Mautino, Ph.D.

# Appendix A

**Data that shows that D1mT has a beneficial antitumor effect in the absence of chemotherapy.**

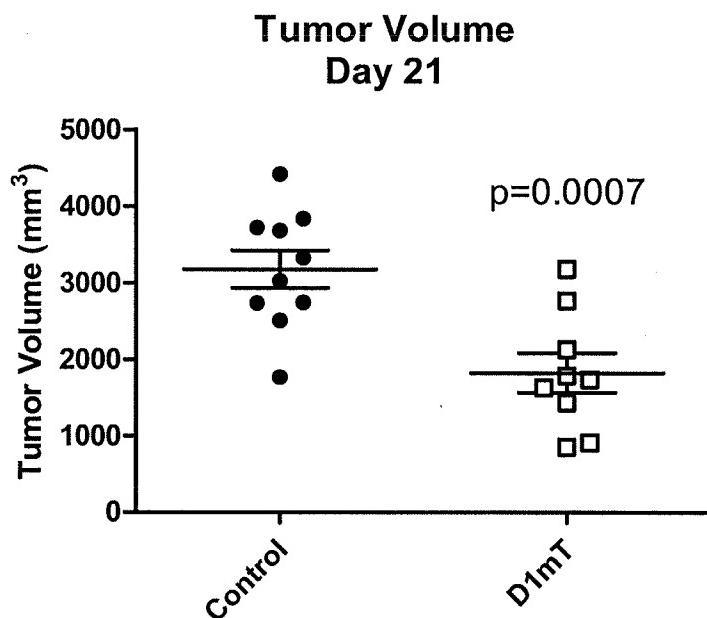
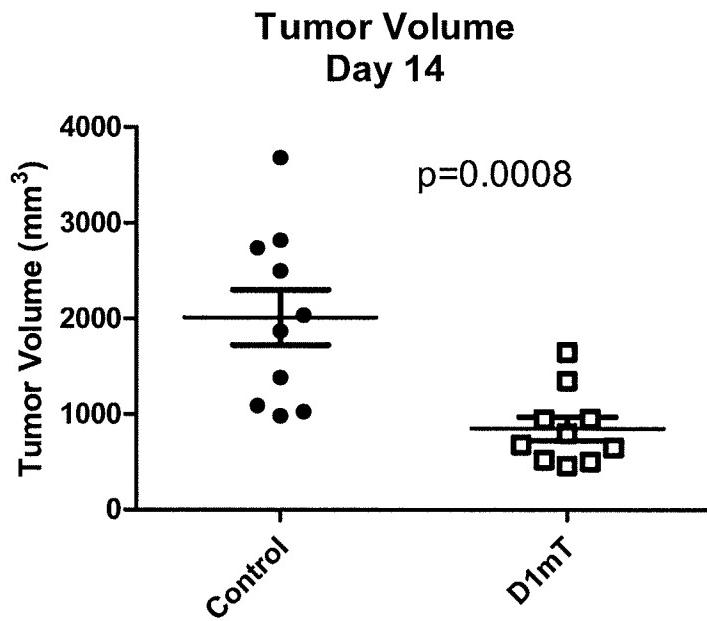
Host mice: B16Bl/6  
Tumor cell line: LLC (Lung)  
Inoculating dose: 300000 cells  
Drug Administration: Starting at day 5 post tumor inoculation, mice received D1mT , at 400 mg/kg, twice a day, via oral gavage, for 3 weekly cycles consisting of 5 days of treatment and 2 days of rest.  
Groups: 10 mice per group

## Survival Curve



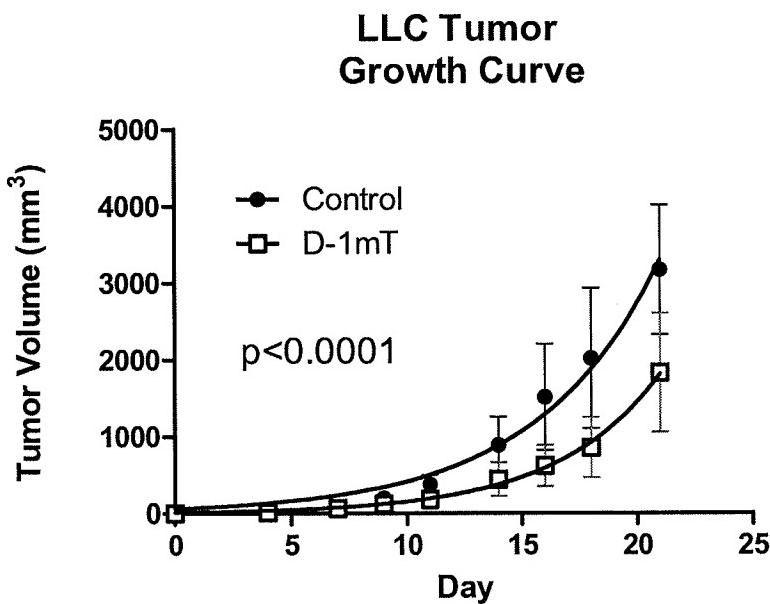
Statistical analysis performed by Log-Rank test

### Analysis of differences in tumor volume



Statistical analysis was performed by a one-tailed t-Test

## Tumor Growth Curves



Statistical analysis was performed by non-linear regression analysis, by fitting the data to an exponential growth model, and then statistically comparing whether the calculated parameters were different between them, using an sum of squares F-test (as recommended by GraphPad Prism 5).

# Inhibition of Indoleamine 2,3-Dioxygenase in Dendritic Cells by Stereoisomers of 1-Methyl-Tryptophan Correlates with Antitumor Responses

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<sup>1</sup>Immunotherapy Center and Departments of <sup>2</sup>Pediatrics, <sup>3</sup>Medicine, and <sup>4</sup>Biostatistics, Medical College of Georgia, Augusta, Georgia; <sup>5</sup>Lankenau Institute for Medical Research, Wynnewood, Pennsylvania; <sup>6</sup>NewLink Genetics Corporation, Ames, Iowa; and <sup>7</sup>Department of Pathology, Anatomy, and Cell Biology, Jefferson Medical College, Philadelphia, Pennsylvania

## Abstract

Indoleamine 2,3-dioxygenase (IDO) is an immunosuppressive enzyme that contributes to tolerance in a number of biological settings. In cancer, IDO activity may help promote acquired tolerance to tumor antigens. The IDO inhibitor 1-methyl-tryptophan is being developed for clinical trials. However, 1-methyl-tryptophan exists in two stereoisomers with potentially different biological properties, and it has been unclear which isomer might be preferable for initial development. In this study, we provide evidence that the *D* and *L* stereoisomers exhibit important cell type-specific variations in activity. The *L* isomer was the more potent inhibitor of IDO activity using the purified enzyme and in HeLa cell-based assays. However, the *D* isomer was significantly more effective in reversing the suppression of T cells created by IDO-expressing dendritic cells, using both human monocyte-derived dendritic cells and murine dendritic cells isolated directly from tumor-draining lymph nodes. *In vivo*, the *D* isomer was more efficacious as an anticancer agent in chemo-immunotherapy regimens using cyclophosphamide, paclitaxel, or gemcitabine, when tested in mouse models of transplantable melanoma and transplantable and autochthonous breast cancer. The *D* isomer of 1-methyl-tryptophan specifically targeted the *IDO* gene because the antitumor effect of *D*-1-methyl-tryptophan was completely lost in mice with a disruption of the *IDO* gene (IDO-knockout mice). Taken together, our findings support the suitability of *D*-1-methyl-tryptophan for human trials aiming to assess the utility of IDO inhibition to block host-mediated immunosuppression and enhance antitumor immunity in the setting of combined chemo-immunotherapy regimens. [Cancer Res 2007;67(2):792–801]

## Introduction

The immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) has been implicated as an immunosuppressive and tolerogenic mechanism contributing to maternal tolerance toward the allogeneic fetus (1), regulation of autoimmune disorders (2–5), and suppression of transplant rejection (6, 7). IDO can also be

expressed by cancer cells in a variety of human malignancies (8, 9). In murine models, transfection of immunogenic tumor cell lines with recombinant IDO renders them immunosuppressive and lethally progressive *in vivo*, even in the face of otherwise protective T-cell immunity (8). In humans, expression of IDO by ovarian and colorectal cancer cells has been found to be a significant predictor of poor prognosis (9, 10).

IDO can also be expressed by host antigen-presenting cells (APC). APCs with the potential to express IDO include human monocyte-derived macrophages (11), human monocyte-derived dendritic cells cultured under specific conditions (12–19), and certain subsets of murine dendritic cells (20–25). In murine tumor models, IDO<sup>+</sup> dendritic cells displaying a plasmacytoid phenotype (CD11c<sup>+</sup>B220<sup>+</sup>) have been found at increased levels in tumor-draining lymph nodes (22). These have been shown to suppress T-cell responses *in vitro* and create antigen-specific T-cell anergy *in vivo* (22, 25). In humans, IDO<sup>+</sup> cells of host origin have been shown in draining lymph nodes of patients with melanoma, breast cancer, and other tumors (13, 22, 26, 27). In patients with malignant melanoma, the presence of these IDO-expressing cells in sentinel lymph node biopsies was correlated with significantly worse clinical outcome (22, 28). Thus, expression of IDO, either by host cells or by tumor cells, seems associated with poor outcome in a number of clinical settings.

These findings have prompted interest in development of IDO inhibitor drugs for cancer immunotherapy (29). The most widely studied of these has been 1-methyl-tryptophan (30–32). Recently, it was shown that 1-methyl-tryptophan displays marked synergy with a number of clinically relevant chemotherapeutic agents when used in combined chemo-immunotherapy regimens (33). In that study, the combination of 1-methyl-tryptophan with cyclophosphamide, cisplatin, doxorubicin, or paclitaxel was able to cause regression of established tumors in a demanding model of autochthonous HER-2/neu-induced murine breast cancers (33). From a clinical standpoint, combining an immunomodulatory agent, such as 1-methyl-tryptophan, with conventional chemotherapy drugs represents an attractive strategy, and a sound mechanistic rationale supporting such chemo-immunotherapy approaches is now being elucidated (34–36).

However, a key unanswered question regarding 1-methyl-tryptophan has been which of the two available stereoisomers (*D* and *L*) should be developed initially for clinical trials. The two isomers differ significantly in their effects on the recombinant IDO enzyme *in vitro* (37), and they could potentially have different biological effects, bioavailability, and off-target toxicities. Most of the studies in the literature have employed the racemic (*D,L*) mixture of 1-methyl-tryptophan comprising both isomers, thus leaving unanswered the question of which stereoisomer would be

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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best suited for use in chemo-immunotherapy regimens. The goal of the present study was to compare the biological activity of the D and L isomers of 1-methyl-tryptophan *in vitro* and *in vivo*, to ask whether their pattern of efficacy *in vitro* correlated with their observed antitumor effect *in vivo*.

## Materials and Methods

**Additional methods available online.** Detailed description of mice, published methods, and statistical analyses are available online at <http://cancerres.aacrjournals.org/>.

**Reagents.** 1-Methyl-D-tryptophan (45,248-3), 1-methyl-L-tryptophan (44,743-9), and 1-methyl-DL-tryptophan (86,064-6) were obtained from Sigma-Aldrich (St. Louis, MO). For *in vitro* use, these were prepared as a 20 mmol/L stock in 0.1 N NaOH, adjusted to pH 7.4, and stored at -20°C protected from light.

**Autochthonous breast cancer model.** Multiparous female MMTV-*Neu* mice, maintained as described (33), have a high incidence of autochthonous mammary gland carcinomas. Tumor-bearing mice were enrolled randomly into experimental groups when tumors reached 0.5 to 1.0 cm in diameter. Tumor volume was measured at the beginning and end of the 2-week treatment period.

**B16F10 and 4T1 tumor models.** B16F10 melanoma (American Type Culture Collection, Manassas, VA) were established in B6 mice by s.c. injection of  $5 \times 10^4$  cultured cells. B78H1-GM-CSF (38), gift of H. Levitsky, (Johns Hopkins University, Baltimore, MD) was implanted by s.c. injection of  $1 \times 10^6$  cells. Orthogonal diameters were measured, and the  $x \cdot y$  product (tumor area) was reported. The use of the orthotopically implanted 4T1 breast cancer line (39) has been described in detail (40). Tumors were implanted by injection of  $1 \times 10^4$  cells in 50  $\mu$ L volume into the mammary fat pad of 6- to 10-week-old BALB/c females. In some experiments, luciferase-transfected 4T1 cells (4T1-luc) were used for bioluminescence imaging, as described in the Supplementary Material.

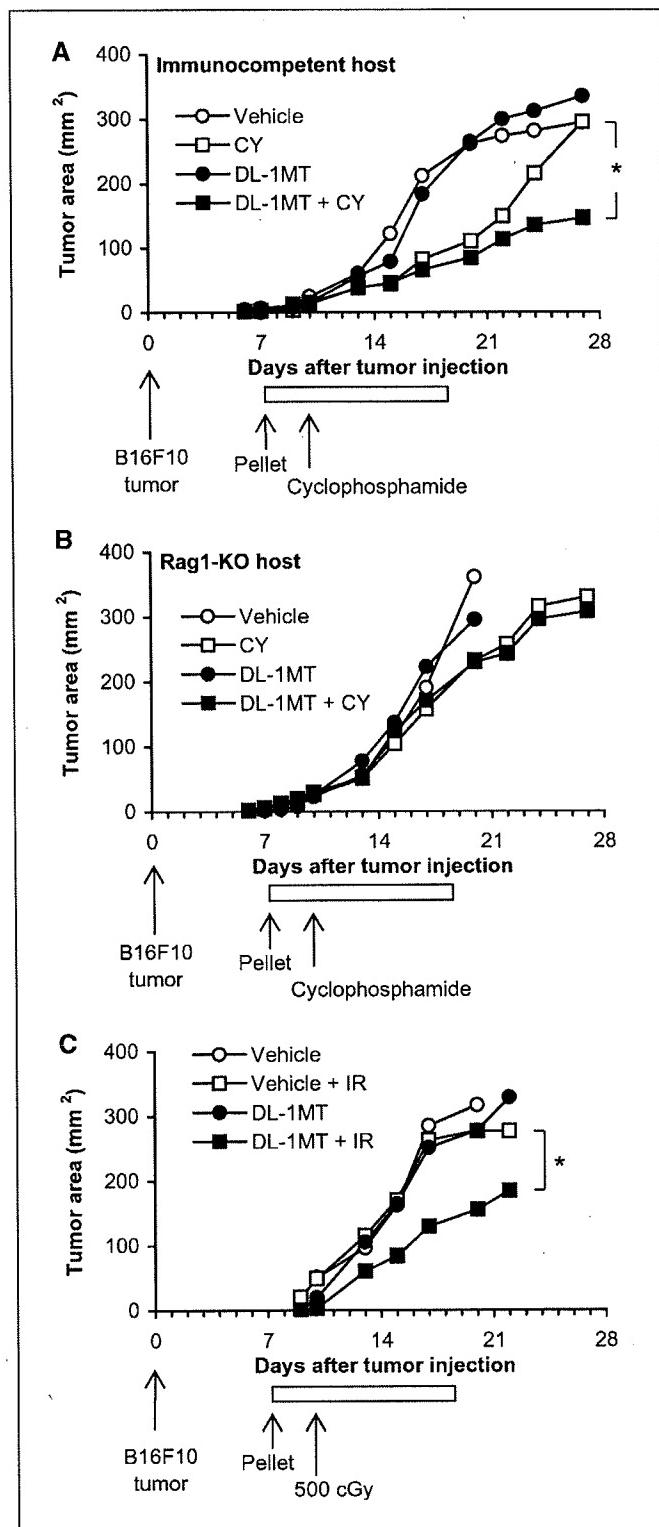
**Administration of 1-methyl-tryptophan and chemotherapeutic agents.** Detailed protocols for administration of 1-methyl-tryptophan, orally and by s.c. pellets, in conjunction with chemotherapy, are given in the Supplementary Material.

**Human and mouse mixed lymphocyte reactions.** Human and murine allogeneic mixed lymphocyte reactions (allo-MLR) were done as detailed in the Supplementary Material and have been previously described (14, 22).

**Western blots.** Western blots were done using affinity-purified polyclonal rabbit antibody against peptides from the NH<sub>2</sub>-terminal and COOH-terminal portion of human IDO, as previously described (13) and as specified in detail in the Supplementary Material.

## Results

**Cooperativity effect of s.c. DL-1-methyl-tryptophan with chemotherapy or radiation in B16F10 melanoma.** We first evaluated the racemic DL mixture of 1-methyl-tryptophan as a component of chemo-immunotherapy using three tumor models: a well-established (day 7) B16F10 melanoma, orthotopically implanted 4T1 breast carcinoma, and autochthonous breast tumors arising in HER-2/neu-transgenic mice. Figure 1A shows established B16F10 tumors treated with DL-1-methyl-tryptophan (20 mg/d by 14-day s.c. copolymer pellet; ref. 1), with or without a single injection of cyclophosphamide (150 mg/kg). DL-1-methyl-tryptophan alone had no effect on tumor growth, and cyclophosphamide alone induced only a transient growth delay. However, the combination of DL-1-methyl-tryptophan + cyclophosphamide resulted in a sustained growth delay and prolonged survival. In all experiments, the end of the study period was defined as the time when all of the mice in the vehicle-only group reached their ethical surrogate end point (tumor area  $\geq 300 \text{ mm}^2$ ). At the point when all mice in the control group had reached this end point, all mice in the



**Figure 1.** Effect of parenteral DL-1-methyl-tryptophan (DL-1MT) in B16F10 tumors. **A**, B16F10 tumors were implanted in syngeneic C57BL/6 mice. Beginning on day 7, mice were treated as shown with timed release s.c. pellets of DL-1-methyl-tryptophan (20 mg/d) plus cyclophosphamide (CY; 150 mg/kg i.p.  $\times$  1 dose). Three identical experiments were done (a representative example is shown), and the pooled results were analyzed in a three-experiment  $\times$  2 group ANOVA. \*,  $P < 0.05$ . **B**, identical experimental design showing that the effect of DL-1-methyl-tryptophan was lost when hosts were immunodeficient Rag1-KO. Groups were not significantly different by ANOVA. **C**, similar experimental design, except that 500 cGy of whole-body cesium-137 irradiation replaced the cyclophosphamide. One of four similar experiments. \*,  $P < 0.05$ , ANOVA.

DL-1-methyl-tryptophan + cyclophosphamide group were still surviving. Figure 1B shows that the effect of DL-1-methyl-tryptophan was lost in immunodeficient Rag1-knockout (Rag1-KO) hosts, indicating that the antitumor effect of DL-1-methyl-tryptophan was entirely immune mediated.

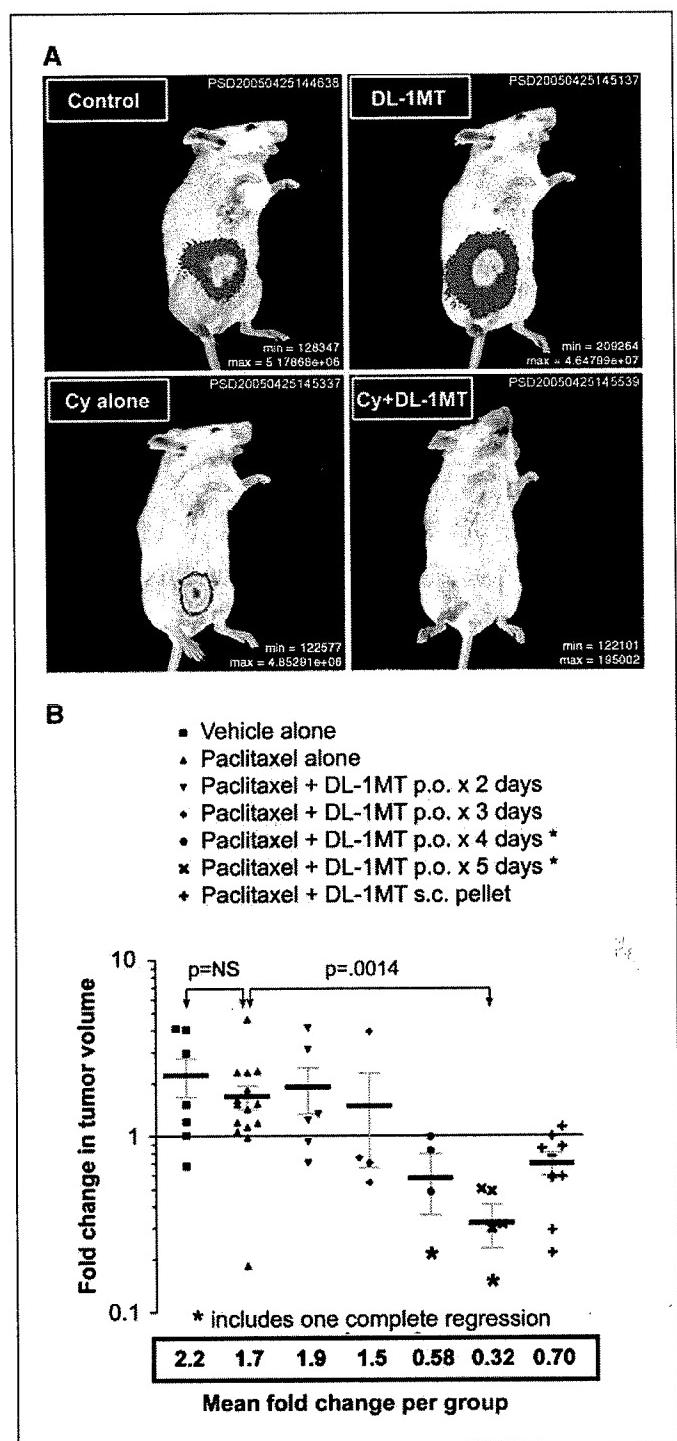
Whole-body irradiation has many of the same effects as chemotherapy when combined with antitumor immunotherapy (41). We tested DL-1-methyl-tryptophan in combination with 500 cGy whole-body irradiation (Fig. 1C). In these experiments, there was considerable variability in the effect of the radiation component alone on

tumor growth, but in all experiments, the effect of DL-1-methyl-tryptophan plus radiation was superior to radiation alone.

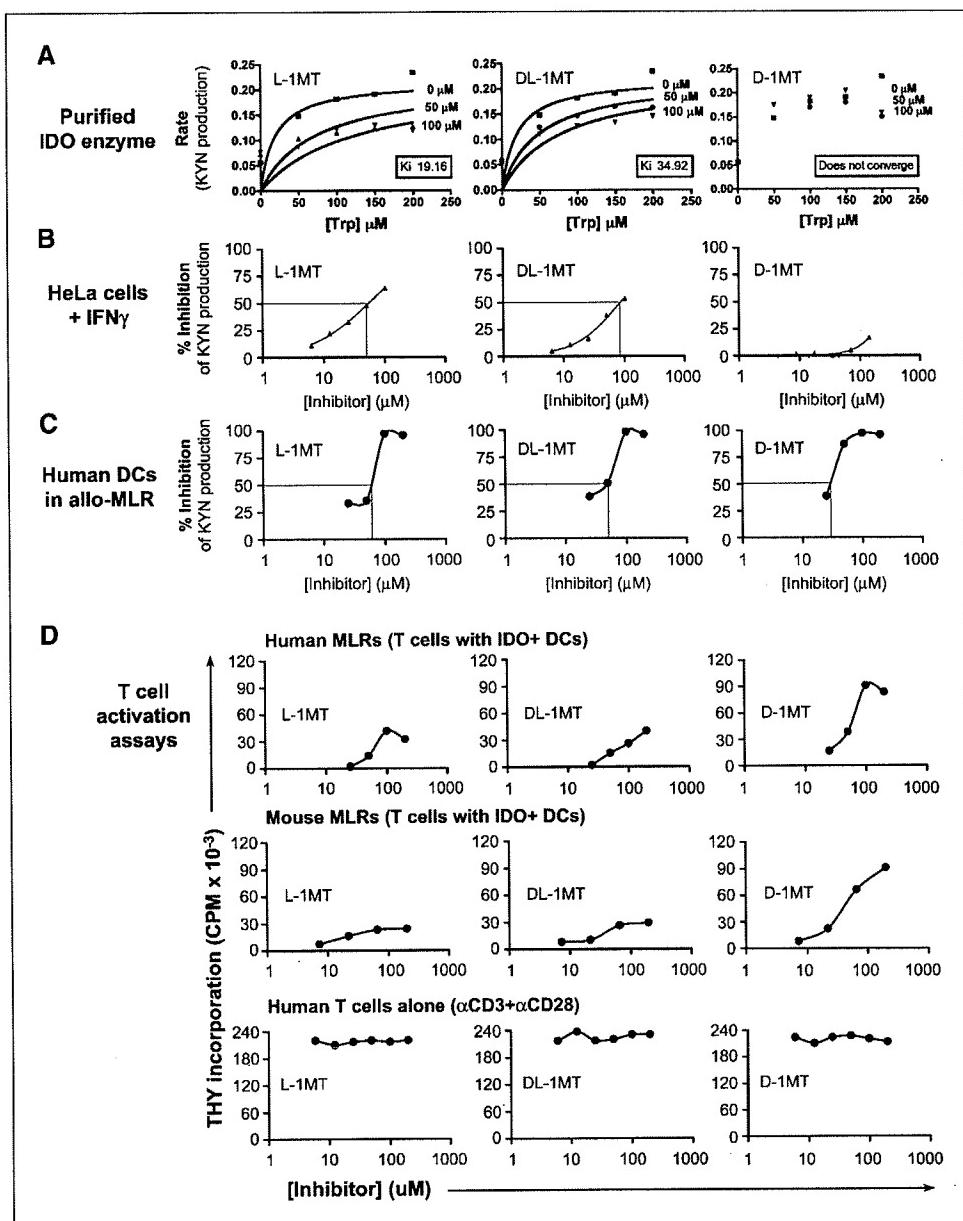
**Cooperativity between oral DL-1-methyl-tryptophan and cyclophosphamide in treating 4T1 breast carcinoma isografts.** We next asked whether DL-1-methyl-tryptophan showed efficacy via the oral route. For these studies, we tested chemo-immunotherapy of the poorly immunogenic 4T1 breast tumor model, implanted orthotopically in mammary tissue of syngeneic hosts. Because orthotopic 4T1 tumors are highly invasive and their margins are difficult to measure conventionally, we followed the tumor size using luciferase-transfected 4T1 (4T1-luc) tumors imaged following luciferin challenge. Oral DL-1-methyl-tryptophan was given by gavage twice daily, five times a week, combined with a weekly single i.p. dose of cyclophosphamide, beginning at the time of tumor implantation. As shown in representative scans in Fig. 2A, cyclophosphamide alone produced a modest reduction in tumor size, but the combination of cyclophosphamide + DL-1-methyl-tryptophan produced a marked decrease in tumor size (survival studies in this model are presented below).

**Oral administration of DL-1-methyl-tryptophan in combination with paclitaxel can elicit regression of autochthonous breast tumors.** We next tested the efficacy of varying durations of oral DL-1-methyl-tryptophan in combination with paclitaxel for the treatment of autochthonous tumors arising in MMTV-Neu mice (33). Mice with tumors were randomly assigned to treatment with paclitaxel for 2 weeks, with or without addition of 2 to 5 days of oral DL-1-methyl-tryptophan during the first week, as indicated in Fig. 2B. Paclitaxel alone caused a minor reduction in the rate of tumor growth, but tumors continued to increase in size during the study period despite paclitaxel. The addition of oral DL-1-methyl-tryptophan produced a progressive reduction in the rate of tumor growth with increasing duration of 1-methyl-tryptophan, such that treatment with 4 and 5 days of DL-1-methyl-tryptophan reversed tumor growth, and caused regression of the established tumors during the treatment period. Five days of administration via the oral route was at least as effective as parenteral delivery of the drug at a comparable daily dose, using implantable s.c. pellet (the last treatment group and the route reported in our previous study; ref. 33).

**In vitro comparison of D versus L isomers of 1-methyl-tryptophan.** We next used *in vitro* models to compare the different isomers of 1-methyl-tryptophan for their biological effects, using two readouts: (a) activity of the IDO enzyme measured as



**Figure 2.** Oral DL-1-methyl-tryptophan in orthotopic 4T1 and autochthonous MMTV-Neu tumors. *A*, orthotopic tumor isografts were established in the mammary fat pad. Treatment was initiated concurrent with tumor challenge, using cyclophosphamide i.p. at 100 mg/kg, once a week and DL-1-methyl-tryptophan oral gavage at 400 mg/kg per dose, twice daily, five times a week. Bioluminescence imaging of 4T1 tumor cell line transfected with luciferase, showing the effect of each treatment on tumor burden. Treatment received by each mouse is indicated. Images were produced at 4 wks following the initiation of treatment. *B*, MMTV-Neu mice bearing 0.5 to 1.0 cm spontaneous tumors were treated for 2 wks with either vehicle alone, paclitaxel alone (13.3 mg/kg i.v. q. M/W/F), or paclitaxel plus oral DL-1-methyl-tryptophan (400 mg/kg i.v. twice daily, given for up to 5 d during the first week, as indicated in the legend). Paclitaxel was given i.v. at over the 2-wk treatment period. The last group received s.c. pellets of 1-methyl-tryptophan, as in Fig. 1. Fold changes in individual tumor volumes over the 2-wk period are plotted for each group. Points, mean fold change for each group (also listed in the box below the graph); bars, SE. \*, fully regressed tumors are included in the calculation of the mean and SE. For the statistical analyses (arrows), the two comparisons of interest were vehicle alone versus paclitaxel alone and paclitaxel alone versus paclitaxel + D-1-methyl-tryptophan × 5 d. Significance was determined at  $P < 0.025$  using a two-group Wilcoxon exact test.



**Figure 3.** Effect of different isomers on *in vitro* enzyme assays and T-cell proliferation. *A*, enzyme kinetics, measured as kynurenine (KYN) production in cell-free assay, for purified recombinant human IDO, showing the effect of the L, DL, and D forms of 1-methyl-tryptophan in the presence of varying concentrations of L-tryptophan substrate. *B*, intracellular IDO enzyme activity (measured as kynurenine production in culture supernatants) by IFN $\gamma$ -activated HeLa cells, showing inhibition by different isomers of 1-methyl-tryptophan. % Inhibition of maximal kynurenine production; lines show interpolated EC<sub>50</sub> for each isomer. *C*, intracellular IDO activity (kynurenine production in MLR supernatants) by human monocyte-derived dendritic cells (DC) activated in allo-MLRs; lines show EC<sub>50</sub>. Combined average of three experiments using three different donors. *D*, effect of 1-methyl-tryptophan isomers on T-cell proliferative responses. Proliferation was measured by thymidine incorporation in allo-MLRs using either human T cells stimulated by IDO-expressing human monocyte-derived dendritic cells (1 of 10 experiments, using a variety of different donor combinations), or mouse T cells stimulated by IDO-expressing plasmacytoid dendritic cells from tumor-draining lymph nodes, as described in Materials and Methods (one of three experiments). As controls, purified human T cells without dendritic cells were activated with immobilized anti-CD3 + anti-CD28 antibodies (one of three experiments).

production of kynurenine from tryptophan and (*b*) a biological readout measured as the ability to prevent the suppression of T-cell proliferation caused by IDO-expressing dendritic cells.

Figure 3*A* shows enzyme kinetics (kynurenine production) using recombinant human IDO enzyme in a cell-free assay system. Using the recombinant enzyme, the L isomer of 1-methyl-tryptophan functioned as a competitive inhibitor ( $K_i = 19 \mu\text{mol/L}$ ), whereas the D isomer was much less effective (no  $K_i$  found at 1-methyl-tryptophan concentrations up to 100  $\mu\text{mol/L}$ ). The DL mixture was intermediate, with a  $K_i$  of 35  $\mu\text{mol/L}$ . These values are consistent with the published literature for studies using cell-free enzyme assays for IDO (37).

We next tested the different isomers in a biological assay, based on the intracellular IDO enzyme expressed by living cells (in this case, HeLa cells activated with IFN $\gamma$ ; Fig. 3*B*). Kynurenine production by HeLa cells showed a pattern of inhibition similar to that of the cell-free recombinant enzyme, with L-1-methyl-

tryptophan being more effective than D-1-methyl-tryptophan. In other studies (data not shown), similar results were obtained using the murine MC57 tumor cell line transfected with recombinant mouse IDO and also the simian COS cell line transfected with human IDO: in each of these transfected cell lines, L-1-methyl-tryptophan was superior to D-1-methyl-tryptophan at inhibiting kynurenine production.

In contrast to the behavior of cell lines, when primary human monocyte-derived dendritic cells were used as the IDO-expressing cells (Fig. 3*C*), the D isomer of 1-methyl-tryptophan was found to be at least as effective as the L isomer in its ability to inhibit IDO activity (measured as kynurenine production in culture supernatants). In these assays, dendritic cells were activated physiologically by exposure to T cells in allo-MLRs, rather than with recombinant IFN $\gamma$ , because we have previously shown that IFN $\gamma$  alone is not sufficient to activate functional IDO in dendritic cells prepared by this protocol (13, 14).

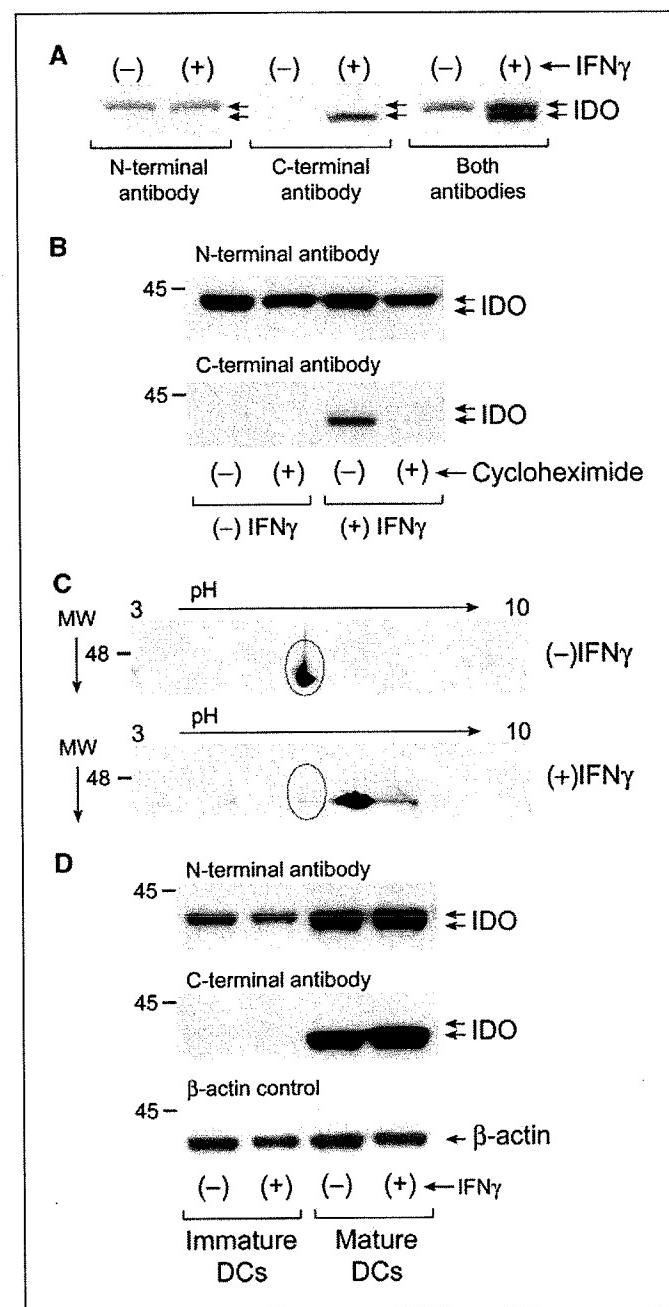
In addition to kynurenine production, we and others have shown that IDO suppresses proliferation of T cells responding to antigens presented by IDO<sup>+</sup> dendritic cells (13, 14, 22). Figure 3D shows a comparison of the different 1-methyl-tryptophan isomers on human T-cell proliferation in allo-MLRs stimulated by IDO<sup>+</sup> monocyte-derived dendritic cells (similar to the MLRs shown in Fig. 3C, but using T-cell proliferation as the readout). Using this readout, the D isomer was found to be reproducibly superior to either the L isomer or the DL mixture, typically eliciting a 2- to 3-fold greater maximum level of T-cell proliferation. A similar pattern was seen using murine T cells (Fig. 3D). For mice, allo-MLRs were done using IDO<sup>+</sup> dendritic cells isolated directly from murine tumor-draining lymph nodes, as previously described (22). These tumor-activated dendritic cells were used to present a constitutive allo-antigen to BM3 TCR-transgenic T cells (specific for the H2K<sup>b</sup> antigen expressed by the C57BL/6 dendritic cells). In this model, just as in the human system, the D isomer of 1-methyl-tryptophan was superior in supporting activation and proliferation of T cells, compared with either the L or DL forms.

To test for nonspecific (off-target) effects of 1-methyl-tryptophan on the T cells themselves, control experiments were done using purified human T cells stimulated by immobilized anti-CD3 + anti-CD28 antibodies (i.e., without any dendritic cells present to express IDO). Under these conditions, none of the 1-methyl-tryptophan preparations had any detectable effect on T-cell proliferation (Fig. 3D). Additional studies (shown in Supplementary Fig. S1) were done further evaluating the D isomer, using MLRs stimulated by dendritic cells derived from mice with a targeted disruption of the *IDO* gene (IDO-KO mice). MLRs using IDO-KO dendritic cells showed that the effects of the D isomer were completely lost when the stimulating dendritic cells lacked IDO. Thus, the D isomer of 1-methyl-tryptophan exerted its effects in MLR specifically by targeting the *IDO* gene expressed by the dendritic cells, not through an off-target effect.

**Western blots suggest the possible existence of more than one isoform of IDO.** The cell type-specific effects of the different isomers of 1-methyl-tryptophan prompted us to ask whether there might be more than one form of IDO expressed in different cells. Published databases suggested potential alternate splicing isoforms of human IDO differing primarily in the COOH-terminal portion of the molecule.<sup>8</sup> Therefore, we generated polyclonal antibodies against peptide sequences in the NH<sub>2</sub>-terminal and COOH-terminal portions of the IDO molecule for use in Western blots, as described in the Supplementary Material.

Figure 4A shows Western blots using the two different antibodies. Samples were prepared from human monocyte-derived macrophages, as a known source of IFN $\gamma$ -inducible IDO (11). As shown in Fig. 4A, the NH<sub>2</sub>-terminal antibody detected a band of ~44 kDa, which was present both before and after IFN $\gamma$  stimulation, and which showed little apparent change with IFN $\gamma$ . In contrast, the COOH-terminal antibody detected an antigen of ~42 kDa, which was only visible after IFN $\gamma$  treatment. A similar pattern of two different constitutive and inducible bands has been described for IDO expression by in other cell types (42). We and others have also shown that IDO can be expressed constitutively at the protein level (e.g., as with the higher molecular weight band)

without necessarily showing enzymatic activity until activated (13, 43). In other experiments (data not shown), HeLa cells showed the same pattern of bands and the same response to IFN $\gamma$ , as did the monocyte-derived macrophages in Fig. 4A.



**Figure 4.** Evidence for two possible isoforms of human IDO. **A**, human monocyte-derived macrophages were prepared as described (11), with or without IFN $\gamma$  treatment for the final 24 h. Lysates were analyzed by Western blot using antibodies against the NH<sub>2</sub>-terminal portion of IDO, the COOH-terminal portion, or a mixture of the two antibodies. All blots were stripped and reprobed for  $\beta$ -actin (data not shown) to confirm even loading. **B**, macrophages, as above, were treated with or without IFN $\gamma$ , in the presence or absence of cycloheximide (10  $\mu$ g/mL).  $\beta$ -Actin blots (data not shown) confirmed even loading. **C**, lysates of macrophages with and without IFN $\gamma$  pretreatment were analyzed by two-dimensional electrophoresis, followed by Western blotting with the NH<sub>2</sub>-terminal-specific anti-IDO antibody. **D**, human monocyte-derived dendritic cells were cultured for 7 d as described in Materials and Methods, with or without addition of a maturation cocktail during the final 48 h. IFN $\gamma$  was added during the last 24 h. Western blots were done as in (B), with the same blot stripped and reprobed for each anti-IDO antibody and the  $\beta$ -actin loading control.

<sup>8</sup>J. Thierry-Mieg et al. AceView: identification and functional annotation of cDNA-supported genes in higher organisms—*Homo sapiens* gene IDO, encoding indoleamine-pyrurole 2,3 dioxygenase. Available from <http://www.ncbi.nlm.nih.gov/IEB/Research/AceView/>.

Figure 4B shows that expression of the IFN $\gamma$ -inducible (lower molecular weight, COOH-terminal) band was blocked by cycloheximide, suggesting that it represented a newly synthesized protein, rather than a posttranslational modification of the larger isoform. Although conventional Western blot analysis did not reveal any obvious change in the larger molecular weight (NH<sub>2</sub>-terminal) isoform in response to IFN $\gamma$ , two-dimensional Western blots (Fig. 4C) revealed that there was a significant IFN $\gamma$ -induced shift in isoelectric point (up to 2 pH units). Thus, these data revealed that both forms of IDO were in fact IFN $\gamma$  responsive, with the larger form appearing to undergo some IFN-induced posttranslational modification, whereas the smaller form seemed to be synthesized *de novo*.

Regulation of IDO activity in dendritic cells is more complex than in macrophages, with multiple factors reported to influence both protein expression and enzymatic activity (17, 19). When we analyzed human monocyte-derived dendritic cells by Western blot (Fig. 4D), there was significant up-regulation of the larger (NH<sub>2</sub>-terminal) isoform with dendritic cell maturation, whereas IFN $\gamma$  treatment had no discernible effect on this band in dendritic cells. The smaller (COOH-terminal) isoform showed no expression in immature dendritic cells and was not inducible in dendritic cells by IFN $\gamma$ . However, the COOH-terminal isoform underwent marked up-regulation with dendritic cell maturation (again independent of IFN $\gamma$ ). Thus, the regulation of the two IDO isoforms in dendritic cells was complex and differed from their regulation in macrophages. However, the essential point was similar for dendritic cells: that more than one species of IDO was present, and that the pattern of expression was regulated by biologically relevant cytokine signals.

**Efficacy of the D isomer of 1-methyl-tryptophan in chemoimmunotherapy.** Based on the superiority of the D isomer in supporting T-cell activation *in vitro*, we tested the D isomer of 1-methyl-tryptophan *in vivo* using the B16F10 model. Established (day 7) B16F10 tumors were treated with cyclophosphamide plus D-1-methyl-tryptophan in a design similar to Fig. 1A. However, in these studies, the dose of the D isomer was reduced 4-fold compared with the dose of the DL mixture used in Fig. 1A, based on its superior efficacy *in vitro*. Even at the lower dose, D-1-methyl-tryptophan + cyclophosphamide showed significant growth delay compared with cyclophosphamide alone (Fig. 5A). Similar results were seen with a second chemotherapeutic agent gemcitabine (Fig. 5B). Neither gemcitabine alone nor D-1-methyl-tryptophan alone had a significant effect on B16F10 tumor growth, but together, the combination produced a significant growth delay.

D-1-methyl-tryptophan had no effect on B16F10 tumors when used as a single agent, but B16F10 is not a highly immunogenic tumor; we therefore asked whether D-1-methyl-tryptophan alone might show an effect if a more immunogenic tumor was used. B78H1-GM-CSF is a subline of B16 that has been transfected with granulocyte macrophage colony-stimulating factor (GM-CSF) to increase recruitment of APCs to the tumor and draining lymph nodes (44). The tumor is modestly immunogenic, although if implanted without irradiation, the tumors invariably grow and kill the host (45). In this somewhat more immunogenic model, D-1-methyl-tryptophan, as a single agent, was found to have a modest but reproducible and statistically significant effect on the growth (Fig. 5C, left). This modest antitumor effect was lost when the hosts were immunodeficient Rag1-KO mice (Fig. 5C, middle), showing that the effect of D-1-methyl-tryptophan was immune mediated. Likewise, the effect of D-1-methyl-tryptophan was lost

when the less immunogenic parental tumor (without GM-CSF) was used in place of B78H1-GM-CSF (Fig. 5C, right). Thus, D-1-methyl-tryptophan did show some modest effect as a single agent when used with an artificially immunogenic tumor. However, this was substantially less potent than the effect of 1-methyl-tryptophan in combination with chemotherapy.

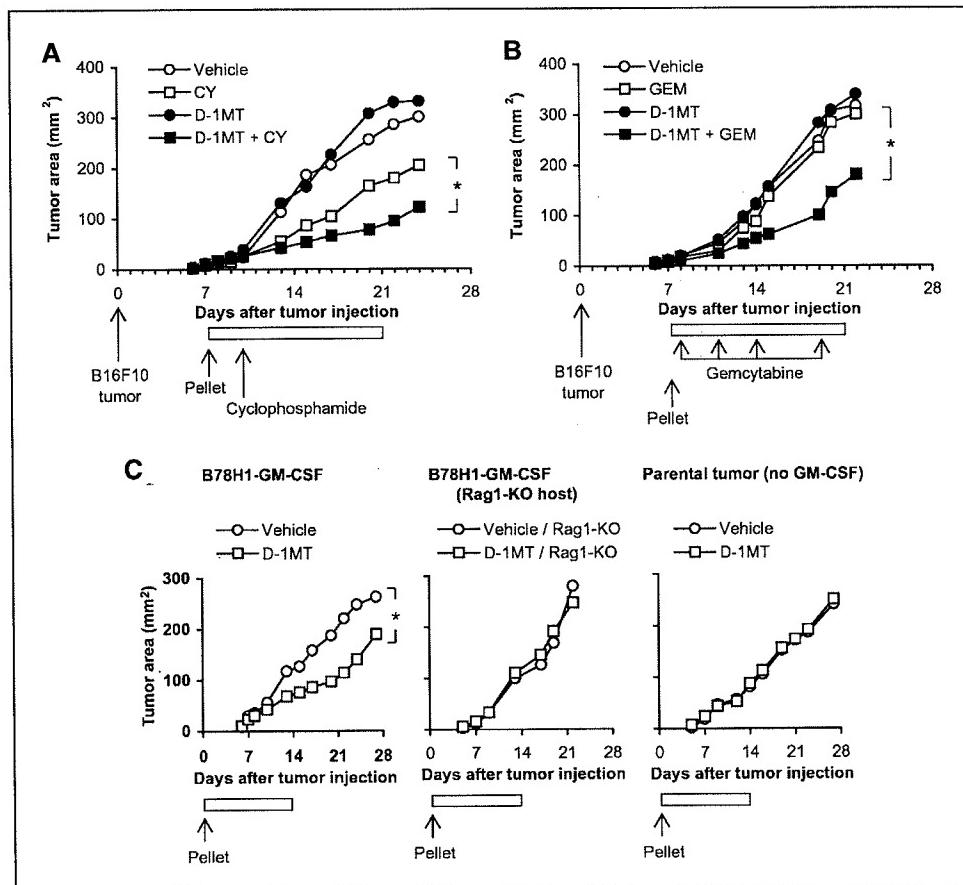
**Comparison of D versus L isomers in chemoimmunotherapy.** We next did side-by-side comparisons of the different isomers of 1-methyl-tryptophan in chemoimmunotherapy regimens. Figure 6A shows a comparison of D versus L versus DL forms of 1-methyl-tryptophan in orthotopic 4T1-luc tumors. Each 1-methyl-tryptophan preparation was given in combination with low-dose cyclophosphamide (25 mg/kg/dose by oral gavage once per week). Although minor effects were observed with the other combinations, only D-1-methyl-tryptophan with cyclophosphamide showed a statistically significant prolongation of survival relative to cyclophosphamide alone (for clarity, these two groups are re-graphed together in the second plot). A second, similar experiment showed the same results, reproducing the survival advantage of D-1-methyl-tryptophan over L-1-methyl-tryptophan in combination with cyclophosphamide.

Figure 6B compares the D versus L isomers of 1-methyl-tryptophan in the autochthonous MMTV-Neu breast tumor model. Both isomers were delivered orally for 5 days, as in Fig. 2C, in combination with paclitaxel. In this model also, D-1-methyl-tryptophan was found to be superior to L-1-methyl-tryptophan (in these studies, the L isomer showed no effect compared with chemotherapy alone).

**Specificity of the D isomer for host IDO *in vivo*.** Finally, one critical outstanding question was the target specificity of the D isomer *in vivo*. We had shown in Supplementary Fig. S1 (Supplementary Material) that the D isomer of 1-methyl-tryptophan specifically targeted the IDO gene *in vitro*. However, it was possible that *in vivo*, D-1-methyl-tryptophan might exert an antitumor effect via some other off-target mechanism. Figure 6C addresses this question by comparing tumors grown in wild-type (IDO sufficient) mice versus tumors grown in IDO-KO mice, each treated with cyclophosphamide + D-1-methyl-tryptophan. The tumors that grew in the IDO-KO hosts would, by definition, have been selected for their lack of dependence on IDO (i.e., they must necessarily be escape variants that could grow in the absence of IDO). Thus, if D-1-methyl-tryptophan truly targeted IDO, then treating tumors grown in IDO-KO mice with D-1-methyl-tryptophan should have no effect on tumor growth; conversely, if D-1-methyl-tryptophan was not specific for IDO, then any off-target effects should be retained in the IDO-KO hosts. Figure 6C shows that tumors grown in IDO-KO mice became completely refractory to the effects of D-1-methyl-tryptophan, thus confirming that IDO was the target of D-1-methyl-tryptophan *in vivo*, as hypothesized. More specifically, these studies suggested that in this model, the relevant target for D-1-methyl-tryptophan was IDO expressed by host cells, rather than by tumor cells, because the tumor cells were the same in both cases.

## Discussion

In the current study, we show significant differences in biological activity between the D and L stereoisomers of 1-methyl-tryptophan. The L isomer was superior at inhibiting activity of purified recombinant IDO enzyme in a cell-free assay and also at inhibiting IDO enzymatic activity in HeLa cells and other cell lines. In



**Figure 5.** Effect of parenteral D-1-methyl-tryptophan in the B16F10 model. *A*, mice with B16F10 tumors were treated in a design similar to Fig. 1*A*, except using the D isomer of 1-methyl-tryptophan at a 4-fold lower dose (5 mg/d by timed release pellets). Cyclophosphamide was given at 150 mg/kg i.p. Three identical experiments were pooled and analyzed by ANOVA.

\* $P \leq 0.05$ . *B*, experimental design similar to (*A*), using gemcitabine 120 mg/kg i.p. on days 8, 11, 14, and 19 following B16F10 tumor implantation. Three experiments were pooled and analyzed by ANOVA.

\* $P < 0.05$ . *C*, B78H1-GM-CSF tumors, or parental tumors without the GM-CSF transgene, were implanted as indicated. Beginning at the time of implantation, mice received 14-day pellets of D-1-methyl-tryptophan (5 mg/d) or vehicle control. *Left*, three experiments were pooled and analyzed by ANOVA.

\* $P = 0.011$ . *Middle*, all hosts were Rag1-KO. *Right*, tumors lacked the GM-CSF transgene (neither of these groups showed significant differences).

contrast, the D isomer was at least as effective as the L isomer at inhibiting IDO enzymatic activity expressed by human or mouse dendritic cells. Unexpectedly, the D isomer was found to be significantly superior to both the L form and the DL mixture when tested by the biologically important readout of T-cell activation in MLRs. *In vivo*, a head-to-head comparison of the antitumor effect of the two isomers showed that the D isomer was more effective than the L isomer, using two different tumors and different chemoimmunotherapy regimens. Thus, the *in vitro* superiority of the D isomer for enhancing T-cell activation in MLRs seemed to correctly predict the superior *in vivo* antitumor efficacy in the models tested, whereas the results of the cell-free enzyme assays did not.

The superiority of the L isomer in the cell-free enzyme assay was expected from the literature (37). However, to our knowledge, no comparison of the two isomers of 1-methyl-tryptophan has been previously reported using assays based on intact cells. Such cell-based systems are important because different cell types may respond differently to the two isomers, as we have now shown. The molecular basis for these cell type-specific differences is not yet known. Possibilities include differential transport into or out of the cells, different subcellular compartmentalization of the inhibitors, or altered metabolism by cellular enzymes. It is also possible that there may be different isoforms of IDO (as could be suggested by our Western blot data), and these might have different sensitivities to the two isomers, although this is currently speculative. Finally, it may be that 1-methyl-tryptophan exerts some of its inhibitory effects on IDO not by competing directly for the catalytic site but by altering enzyme activity in another way that does not register in the cell-free enzyme assay.

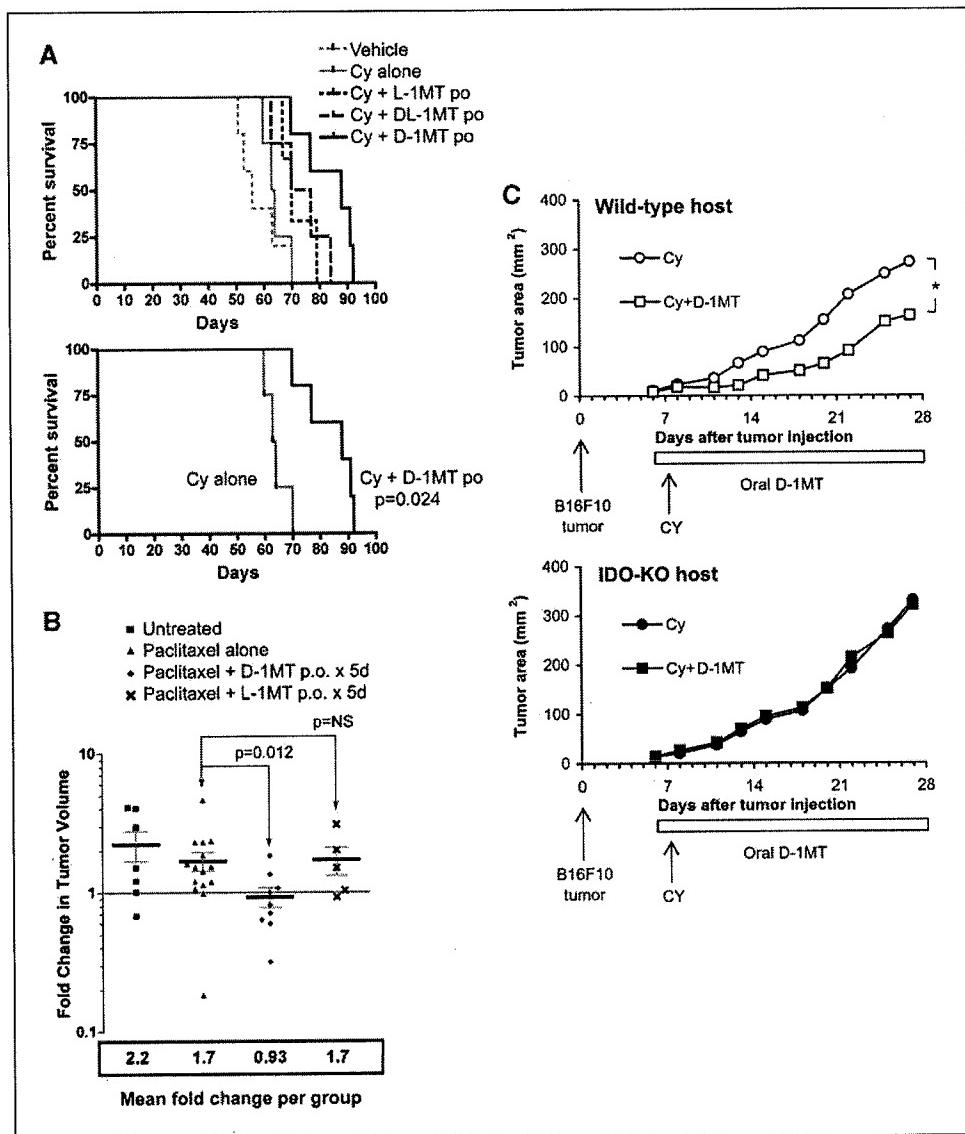
Others have also reported efficacy of the D isomer of 1-methyl-tryptophan for enhancing T-cell responses *in vitro* and *in vivo* (46, 47). Importantly, our data unambiguously showed that the T cell-enhancing effect of D-1-methyl-tryptophan *in vitro* was completely lost when APCs were derived from IDO-KO mice; and, likewise, the antitumor efficacy of D-1-methyl-tryptophan *in vivo* was lost when the tumor-bearing hosts were IDO-KO. Thus, the molecular target of D-1-methyl-tryptophan was indeed IDO, and the efficacy of D-1-methyl-tryptophan was not due to some off-target effect. This would also be consistent with recent studies using RNA-knock-down techniques, which concluded that the major molecular target of the DL-mixture of 1-methyl-tryptophan was IDO, rather than an off-target effect (48).

One critical reason underlying the superior activity of the D isomer *in vivo* may be our observation that the L isomer seemed actively inhibitory for T-cell activation in MLRs. Both isomers were equally effective at blocking the enzymatic activity of IDO in MLRs (measured as kynurenine production in the supernatant); yet, the L isomer could not produce the same high levels of T-cell proliferation achieved by the D isomer. Revealingly, the DL mixture also proved less effective than the D isomer alone, suggesting that the presence of the L isomer actively inhibited T-cell proliferation. The nature of this inhibition is currently unknown. However, it did not seem to be due to a direct toxic effect of L-1-methyl-tryptophan on the T cells themselves because T cells stimulated by mitogen (i.e., in the absence of IDO-expressing dendritic cells) were no longer affected by L-1-methyl-tryptophan. This suggests that the off-target inhibitory effect of the L isomer might be due to a toxic effect of L-1-methyl-tryptophan on the IDO-expressing dendritic cell itself.

(e.g., rendering it less able to present antigen to the T cells). Perhaps consistent with such an off-target effect on dendritic cells, it has recently been reported that exposure of dendritic cells *in vitro* to the DL-mixture of 1-methyl-tryptophan at 1,000  $\mu\text{mol/L}$  (much higher than the maximum concentration used in the current study) caused alteration in dendritic cell function, which did not seem related to the effect of DL-1-methyl-tryptophan on IDO itself (49). Alternatively, the T cells might be sensitive to some metabolite of the L isomer

generated by the dendritic cells. In either case, it seems that the D isomer of 1-methyl-tryptophan escaped this off-target inhibitory effect on T-cell activation, perhaps precisely because it was not the "natural" stereoisomer.

Although the D isomer showed superior efficacy in our chemoimmunotherapy models, the L isomer proved better at inhibiting IDO in HeLa cells and in mouse tumor cell lines transfected with IDO. Thus, it may be that in certain biological contexts the L isomer



**Figure 6.** D-1-methyl-tryptophan provides greater survival benefit in combination therapy, in an IDO-dependent fashion. *A*, 4T1-luc orthotopic isografts were established in the mammary fat pad. Cyclophosphamide was given at 25 mg/kg orally once a week, and 1-methyl-tryptophan (D, L, or DL) given at 400 mg/kg by oral gavage twice daily, five times a week by gavage, beginning at the time of tumor implantation. *Top*, time to endpoint for all groups; *bottom*, only the cyclophosphamide versus cyclophosphamide + D-1-methyl-tryptophan groups, for clarity. The comparisons of interest were between D-1-methyl-tryptophan + cyclophosphamide versus cyclophosphamide and L-1-methyl-tryptophan + cyclophosphamide versus cyclophosphamide. Because survival data were not censored, groups were analyzed using a two-group Wilcoxon exact test; statistical significance was determined at  $P < 0.025$ . The combination of D-1-methyl-tryptophan + cyclophosphamide showed a significant survival benefit over cyclophosphamide alone ( $P = 0.024$ ), whereas L-1-methyl-tryptophan + cyclophosphamide was not different from cyclophosphamide alone ( $P = 0.14$ ). *B*, MMTV-Neu mice with tumors were treated for 2 wks as in Fig. 2B, receiving either vehicle alone, paclitaxel alone, or paclitaxel (13.3 mg/kg q. MWF) plus oral D-1-methyl-tryptophan or L-1-methyl-tryptophan for 5 d, as indicated. For statistical analysis, the comparisons of interest were D-1-methyl-tryptophan + paclitaxel versus paclitaxel alone and L-1-methyl-tryptophan + paclitaxel versus paclitaxel alone. Significance was determined at  $P < 0.025$  using a two-group Wilcoxon exact test. The fold change of the D-1-methyl-tryptophan + paclitaxel group was significantly smaller than that of paclitaxel alone ( $P = 0.012$ ), whereas paclitaxel + L-1-methyl-tryptophan was not different from paclitaxel alone ( $P = 0.85$ ). *C*, effects of the D isomer of 1-methyl-tryptophan require an intact host IDO gene. B16F10 tumors were grown in either wild-type B6 hosts or IDO-KO hosts on the B6 background. All groups received cyclophosphamide, with or without oral D-1-methyl-tryptophan (2 mg/mL in drinking water). Analysis by ANOVA showed that cyclophosphamide + D-1-methyl-tryptophan was significantly different (\*,  $P < 0.05$ ) than cyclophosphamide alone for the wild-type hosts, but there was no effect of D-1-methyl-tryptophan when tumors were grown in IDO-KO hosts.

might be preferable, whereas in other contexts, the D isomer is superior. This might become relevant where the target of 1-methyl-tryptophan is IDO expressed by the tumor cells themselves, rather than by host dendritic cells. However, the data from our *in vitro* T-cell activation models and from our *in vivo* chemoimmunotherapy models suggest that in these systems, the beneficial effect of the D isomer on T-cell activation is the key advantage, rendering the D isomer superior in these settings. Furthermore, based on the fact that efficacy of D-1-methyl-tryptophan was lost when the host mice were genetically deficient in IDO (Fig. 6C), our data suggest that the molecular target of D-1-methyl-tryptophan in our system was the IDO activity expressed specifically by host APCs, not by the tumor cells themselves.

In the murine models used in this study, relatively high doses of 1-methyl-tryptophan were required to see an antitumor effect. However, this seems to represent a peculiarity of 1-methyl-tryptophan pharmacokinetics in mice. Preclinical pharmacology studies in both rats and canines (to be published elsewhere) show that these animals require significantly lower doses per kilogram to achieve plasma levels in the same range. These lower doses should be readily achievable clinically.

The combination of 1-methyl-tryptophan with chemotherapy (cyclophosphamide, paclitaxel or gemcitabine) was more potent against established tumors than either 1-methyl-tryptophan or chemotherapy alone. Regimens featuring chemotherapy plus immunotherapy are receiving increasing attention (34, 35). In part, this is because they are readily applicable in the clinic because patients do not have to be denied standard chemotherapeutic agents to receive immunotherapy. In addition, there is a sound mechanistic rationale underlying combined chemo-immunotherapy. Chemotherapy causes death of tumor cells, thus releasing tumor antigens into the host antigen-presentation pathway (34).

In addition, certain chemotherapy drugs seem to decrease the number and activity of regulatory T cells (50, 51), which may assist the immunotherapy regimens in breaking tolerance to tumor antigens. Finally, the recovery phase from chemotherapy-induced lymphopenia seems to constitute a favorable window for reactivating previously tolerized T cells (41). However, despite these effects, chemotherapy alone does not elicit an effective antitumor immune response. We hypothesize that one reason for this failure is because the antigens released by chemotherapy are presented first in the tumor-draining lymph nodes. We and others have previously shown that tumor-draining lymph nodes are a highly tolerogenic microenvironment (52), due at least in part to the presence of IDO-expressing APCs (22, 25). Thus, IDO<sup>+</sup> host APCs may play an important pathogenic role in helping the tumor re-establish immunologic tolerance toward itself after it is disrupted by chemotherapy. Based on our current data, we hypothesize that the addition of an IDO inhibitor drug during this post-chemotherapy period may allow the tumor-bearing host to mount an effective immune response to tumor antigens during this post-chemotherapy window of opportunity.

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